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## In vitro fermentation of fibrous substrates by water buffalo ruminal cellulolytic bacteria consortia

Fermentación *in vitro* de consorcios bacterianos celulolíticos ruminales de búfalos de agua en sustratos fibrosos

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### ABSTRACT:

**Objective.** To measure the *in vitro* fermentation variables of a cellulolytic bacteria consortium (CBC) isolated from a water buffalo rumen in coculture with total ruminal bacteria (TRB) on two fibrous substrates. **Materials and methods.** A CBC was isolated from the ruminal fluid of a female water buffalo in selective cellulolytic media. The experimental design was completely random with a 3x2 factorial arrangement; factors were treatments [TRB, CBC, and coculture (TRB + CBC)] and substrates (cobra grass and corn stover). Total gas and methane (CH<sub>4</sub>) production were measured at different time intervals. At 72 h, measurements were taken of pH, ammoniacal nitrogen (NH<sub>3</sub>-N), dry matter degradation (DMD), neutral detergent fiber degradation (NDFD) and total bacteria population. **Results.** Gas production with both substrates was highest ( $p \leq 0.05$ ) in the coculture at 3, 6 and 24 h. At 48 and 72 h, gas production in the cobra grass was highest ( $p \leq 0.05$ ) in the coculture. The coculture and TRB did not differ ( $p > 0.05$ ) in terms of CH<sub>4</sub>, DMD and NDFD values at 48 and 72 h. With the cobra grass, NH<sub>3</sub>-N concentration was higher ( $p \leq 0.05$ ) in the coculture than in the TRB. **Conclusions.** The gas production and dry matter degradation values of the water buffalo rumen cellulolytic bacteria consortia indicate them to be a promising alternative for improving cobra grass structural carbohydrates degradation when in coculture with bovine ruminal bacteria.

**KEYWORDS:** Ruminal bacteria, Bubalus bubalis, cellulosic coculture, degradation.

### RESUMEN:

**Objetivo.** Determinar las variables fermentativas *in vitro* de un consorcio bacteriano celulolítico (CBC) aislado de una búfala de agua en cocultivo con bacterias ruminales totales (BRT) en sustratos fibrosos. **Materiales y métodos.** Un CBC se aisló de fluido ruminal de una búfala de agua en medios selectivos celulolíticos. El diseño experimental fue un diseño completamente al azar con

arreglo factorial 3x2, los factores fueron tratamientos [BRT, CBC y un cocultivo (BRT + CBC)] y sustratos (pasto pangola y rastrojo de maíz). La producción de gas total y metano (CH<sub>4</sub>) se midieron a diferentes intervalos de tiempo. Además, se estimó pH, nitrógeno amoniacal (N-NH<sub>3</sub>), degradación de materia seca (DMS) y de fibra detergente neutro (DFDN), y la población de bacterias totales a 72 h de incubación. **Resultados.** El cocultivo produjo mayor ( $p \leq 0.05$ ) cantidad de gas a las 3, 6 y 24 h en ambos sustratos. A las 48 y 72 h, el cocultivo produjo mayor ( $p \leq 0.05$ ) gas en pasto cobra. El cocultivo y las BRT no presentaron diferencias ( $p > 0.05$ ) en la producción de CH<sub>4</sub> a 48 y 72 h, y en DMS y DFDN ( $p > 0.05$ ). En el pasto cobra, la concentración de N-NH<sub>3</sub> con el cocultivo fue mayor ( $p \leq 0.05$ ) que con BRT. **Conclusiones.** La producción de gas y degradación de materia seca de los consorcios bacterianos celulolíticos procedentes del rumen de una búfala de agua muestran que son una alternativa para mejorar la fermentación de carbohidratos estructurales del pasto cobra cuando se cocultivan con bacterias ruminales bovinas.

**PALABRAS CLAVE:** Bacterias ruminales, *Bubalus bubalis*, celulósicos, cocultivo, degradación.

## INTRODUCTION

The dry tropics of Mexico contain approximately 51 million hectares of pasture, which represent a substantial source of forage for ruminants. These forages are characterized by their rapid growth and early maturation, leading to high concentrations of cellulose, hemicellulose and lignin (1).

The ruminal microorganisms of water buffalo (*Bubalus bubalis*) differ from those of cattle (*Bos indicus* and *B. taurus*), and are better adapted to tropical forages. Water buffalo are therefore better able to synthesize microbial protein due to more efficient fermentation of structural carbohydrates. They can transform low quality forages into available energy in the form of volatile fatty acids (2,3), and can ruminate 50% more than cattle (3). This difference may be due to the higher cellulolytic bacteria and ciliated protozoa populations in the water buffalo rumen compared to that of cattle (2). For example, water buffalo have substantially higher proportions of protozoa in the subfamilies *Diplodiniinae* (28.6 vs. 1.4%) and *Epidinium* (5.3 vs. 0.0%) (4), and the buffalo bacterial population is  $0.60 \log^{10}$  cells  $g^{-1}$  larger than that of cattle (5). This discrepancy has fostered interest in understanding and manipulating the water buffalo microorganism community (3).

Microorganisms generally exist in diverse and complex communities known as consortia (6,7), which interact with their environment (8). Communication between microorganisms is via direct cell-cell interactions, metabolites or molecular signals (6). Cellulolytic consortia are very efficient at degrading cellulose and hemicellulose (7), and can be used as additives in ruminant feed to improve cellulose and hemicellulose degradation in forages. The present study objective was to estimate the *in vitro* fermentation characteristics of cellulolytic bacterial consortia collected from the rumen of a female water buffalo (*Bubalus bubalis*) in coculture with ruminal bacteria from a female Swiss-bu (Brown Swiss x Brahman) calf using cobra grass (*Brachiaria sp.*) and corn (*Zea mays*) stover as substrates.

## MATERIALS AND METHODS

**Study site.** The study was done at the Animal Nutrition Laboratory of the Faculty of Veterinary Medicine and Zootechny No. 2, Autonomous University of Guerrero (Cuajinicuilapa, Guerrero, Mexico). Located in southwest Mexico (16°08' N; 98°23' W; 50 m asl), regional climate is warm subhumid with summer rains, average rainfall is 1,200 mm and average annual temperature is 25°C (9).

**Ethical aspects.** Both the water buffalo and Swiss-bu calf were handled following the internal bioethical and well-being guidelines of the Autonomous University of Guerrero, which comply with established federal guidelines (NOM-062-ZOO-1999) (10).

**Culture medium.** Medium components were 30 mL clarified ruminal fluid [fresh bovine ruminal liquid centrifuged at  $12,857 \times g$  for 10 min and sterilized (All American ° 1941X, USA) for 15 min at 121°C and 15 psi]; 5 mL mineral I solution [6 g K<sub>2</sub>HPO<sub>4</sub> (Sigma-Aldrich °) in 1000 mL distilled water]; 5 mL mineral II solution [6 g KH<sub>2</sub>PO<sub>4</sub> (Sigma-Aldrich °) + 6 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Merck °) + 12 g NaCl (Sigma-Aldrich °)]

+ 2.45 g MgSO<sub>4</sub> (Sigma-Aldrich<sup>®</sup>) + 1.6 g CaCl<sub>2</sub>·2H<sub>2</sub>O (Sigma-Aldrich<sup>®</sup>) in 1000 mL distilled water]; 0.1 mL 0.1% resazurin (Sigma-Aldrich<sup>®</sup>); 0.2 g soy peptone (Merck<sup>®</sup>); 0.1 g yeast extract (Sigma-Aldrich<sup>®</sup>); 2 mL cysteine-sulfide solution [2.5 g L-cysteine (Sigma-Aldrich<sup>®</sup>) in 15 mL 2N NaOH (Meyer<sup>®</sup>) + 2.5 g Na<sub>2</sub>S·9H<sub>2</sub>O (Merck<sup>®</sup>) diluted in 1000 mL distilled water]; 5 mL of an 8% Na<sub>2</sub>CO<sub>3</sub> solution (Merck<sup>®</sup>) and 52.6 mL distilled water. The medium was sterilized for 15 min in an autoclave at 121°C and 15 psi (11).

**Cellulolytic bacterial consortium.** Before sample collection, the water buffalo (450 Kg PV) was kept in a pangola grass (*Digitaria decumbes*) pasture, without supplements. Ruminant fluid was extracted from the buffalo (*Bubalus bubalis*) with an esophageal probe and centrifuged at 1.157 x g for 3 min (Metrix Velocity 14, USA). The supernatant was recovered and, under a biosecurity hood (Labconco<sup>®</sup>, USA), used as an inoculum. Sterile culture medium (9 mL) was added to test tubes (Pirex<sup>®</sup>, Mexico; 18x150 mm) containing a sterile strip (3x30 mm) of Whatman<sup>®</sup> paper. The tubes were kept under CO<sub>2</sub> flow in an incubator (Ecoshel 9082, Mexico) at 39°C for 24 h to verify sterility. Using three replicates, 1 mL inoculum was added to a test tube containing medium and sterile Whatman<sup>®</sup> paper, and incubated at 39°C until the paper was observed to degrade; 1 mL of this inoculum was transferred to another tube containing medium and sterile Whatman<sup>®</sup> paper and again incubated at 39°C until the paper degraded. After three transferences, a B inoculum was produced. Sterile medium (9 mL) was placed in tubes (18x150 mm) containing 0.05 g sterile crystalline cellulose (Sigma-Aldrich<sup>®</sup>), and the tubes placed under CO<sub>2</sub> flow at 39°C for 24 h to verify sterility. In triplicate, a tube containing crystalline cellulose was inoculated with 1 mL B inoculum and kept at 39°C for 72 h; 1 mL of the resulting inoculated medium was transferred to another sterile tube and incubated at 39°C for 72 h. Four of these transferences were done to produce a cellulolytic bacterial consortium capable of degrading Whatman<sup>®</sup> paper and crystalline cellulose.

**Treatment.** Three treatments were tested. 1) TRB = 5 mL total ruminal bacteria extracted from the ruminal fluid of a female Swiss-bu (Brown Swiss x Brahman) calf fitted with a ruminal canula; the calf had been kept in a pasture of pangola. The ruminal fluid was centrifuged at 1,157 g for 3 min to precipitate protozoa and fiber particles (11). 2) CBC = 5 mL of a cellulolytic bacteria consortium from water buffalo grown in culture medium containing cellobiose (0.2 g 100 mL<sup>-1</sup> medium; Sigma-Aldrich<sup>®</sup>). 3) Coculture = 5 mL TRB + 5 mL CBC; total bacterial population in the TBR and CBC was measured by direct count with a Petroff-Hausser camera (Hausser #39000, Electron Microscopy Sciences, USA) and a microscope (BX31, Olympus, USA) at 1000x magnification. Bacterial population was calculated with the formula: number of bacteria = (quadrant average)(dilution factor, 2X10<sup>7</sup>) (11).

**Substrate.** Cobra grass (*Brachiaria hybrid* CV. CIAT BR02/1794) harvested 56 days post-resprout and corn stover were the two tested substrates. Both were dehydrated at 60 °C for 72 h in a drying oven (Felisa<sup>®</sup> FE-293A, Mexico), ground (Thomas-Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) and screened through 1 mm mesh. Substrate crude protein (CP), ash (AS) and organic matter (OM) content were measured following AOAC methods (12). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured with the ANKOM Technology Method according to Van Soest et al (13)(Table 1).

TABLE 1

**Table 1.** Chemical composition (% dry base) of cobra grass and corn stover substrates.

Substrate	CP	NDF	ADF	Hemicellulose	AS	OM
Cobra Grass	7.82	69.05	47.96	12.09	12.14	87.86
Corn stover	3.86	77.20	46.92	30.28	12.31	87.69

CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; As = ash; OM = organic matter.

**In vitro gas production.** Glass serological vials (120 mL) were filled with 0.5 g substrate and 45 mL culture medium, each being considered a biodigester. These were sterilized for 15 min at 121°C and 15 psi, and incubated at 39°C for 24 h to verify sterility. Using four repetitions per treatment, the biodigesters were inoculated with TRB, CBC or the coculture and incubated at 39°C for 72 h. At seven incubation times (3, 6, 9, 12, 24, 48 and 72 h) (14), gas production was measured by displacement of the plunger in a glass syringe (50 mL; BD Yale<sup>®</sup>, Brazil).

**Methane (CH<sub>4</sub>) production.** This parameter was measured following a modified version of Stolaroff et al (15). Hypodermic needles (20 G x 32 mm) were attached to the ends of a Taygon<sup>®</sup> hose (2.38 mm internal Ø x 45 cm long), and used to connect the biodigesters to trap vials filled with a 2 N NaOH solution [80 g NaOH (Merck<sup>®</sup>) in 1000 mL distilled water]. Methane production was quantified as mL of displaced NaOH solution at 24, 48 and 72 h incubation.

**pH.** This parameter was measured with a potentiometer (Hanna<sup>®</sup> HI2211, Italy; calibration: pH 7 and 4) at 72 h incubation.

**Total bacterial count.** At 72 h incubation, 1 mL of medium was collected from the center of the biodigester, and placed in a test tube with 0.25 mL 10% formaldehyde (Sigma-Aldrich<sup>®</sup>). Total bacterial count was calculated by direct count in a Petroff-Hausser chamber (11).

**Ammoniacal nitrogen (NH<sub>3</sub>-N).** A sample of medium (1 mL) from the biodigester was mixed with 0.25 mL 25% metaphosphoric acid (4:1 ratio), centrifuged at 3,500 x g for 25 min and the supernatant recovered in 2 mL vials. Supernatant (20 µL) was mixed with 1 mL phenol solution [10 mg Na<sub>2</sub>(NO)Fe(CN)<sub>5</sub>.H<sub>2</sub>O (Meyer<sup>®</sup>) + 10 g phenol crystals (Meyer<sup>®</sup>) diluted in 1000 mL distilled water] and 1 mL hypochlorite solution [7.5 g NaOH (Reasol<sup>®</sup>) + 21.3 g Na<sub>2</sub>HPO<sub>4</sub> (Meyer<sup>®</sup>) + 15 mL hypochlorite (5%; Reasol<sup>®</sup>) diluted to 1000 mL with distilled water]. This mixture was incubated for 30 min at 37 °C in a water bath. Distilled water (5 mL) was added to the mixture and it was agitated in a vortex (Genie 2 G-560, USA). Absorbance was measured at 630 nm in a UV-VIS spectrophotometer (Jenway<sup>®</sup> 6850, USA) calibrated (r<sub>2</sub> = 0.9994) with an ammoniacal nitrogen concentration method (16).

**Degradation of dry matter (DMD) and neutral detergent fiber (NDFD).** The sample remaining in the biodigester was filtered using ANKOM<sup>®</sup> 541 bags to a constant weight. Bags containing samples were dried for 24 h at 60°C in a drying oven. Dry matter degradation was calculated with the formula DMD(%) = (initial sample – residual sample / initial sample) \* 100 (14). The bags were then heat sealed and NDF content measured (13). The percentage of NDFD was calculated with the formula NDFD(%)=(initial NDF – residual NDF / initial NDF)\*100 (14).

**Results analysis.** A completely random 3x2 factor analysis was applied using the three treatments (i.e. CBC, TRB and Coculture) and two substrates (i.e. cobra grass and corn stover).

The statistical model was

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ijk}; \text{ where}$$

$Y_{ijk}$  = response variable;  $\mu$  = general mean;

$A_i$  = effect of treatment;  $B_j$  = effect of substrate;  $AB_{ij}$  = effect of treatment / substrate interaction;  $\varepsilon_{ijk}$  = random error.

Data were analyzed with the GLM procedure in the SAS<sup>®</sup> package (17). Means were adjusted by least means using LSMEANS in SAS<sup>®</sup> (17), and compared with the Tukey test (p≤0.05).

## RESULTS

With cobra grass, gas production at 3, 6, 24, 48 and 72 h was highest (p≤0.05) in the coculture versus the CAC and TRB treatments; the same was true with the corn stover at 3, 6, 9, 12 and 24 h (Table 2). At 48

and 72 h, no difference ( $p > 0.05$ ) in gas production was observed between the coculture and TRB with corn stover. At these same times the coculture in cobra grass produced more ( $p \leq 0.05$ ) gas than TRB and CBC (Table 2).

TABLE 2

**Table 2.** Gas volume ( $\text{mL g}^{-1} \text{DM}$ ) accumulated during *in vitro* fermentation of cobra grass and corn stover inoculated with a cellulolytic bacteria consortium from water buffalo, total ruminal bacteria from a cow and a coculture of these communities.

Incubation time (h)	Cobra Grass			Corn stover			SME
	CBC	Coculture	TRB	CBC	Coculture	TRB	
3	51.6 <sup>b</sup>	59.7 <sup>a</sup>	48.9 <sup>b</sup>	63.3 <sup>a</sup>	51.7 <sup>b</sup>	31.7 <sup>c</sup>	2.02
6	61.7 <sup>c</sup>	70.5 <sup>b</sup>	59.1 <sup>c</sup>	84.3 <sup>a</sup>	83.8 <sup>a</sup>	62.3 <sup>c</sup>	2.13
9	72.5 <sup>c</sup>	83.7 <sup>b</sup>	81.6 <sup>b</sup>	90.7 <sup>a</sup>	90.1 <sup>a</sup>	79.2 <sup>b</sup>	2.20
12	76.0 <sup>c</sup>	102.6 <sup>a</sup>	106.6 <sup>a</sup>	96.5 <sup>b</sup>	107.6 <sup>a</sup>	94.5 <sup>b</sup>	1.36
24	82.7 <sup>d</sup>	145.4 <sup>a</sup>	132.5 <sup>b</sup>	101.7 <sup>c</sup>	150.8 <sup>a</sup>	133.0 <sup>b</sup>	2.26
48	93.9 <sup>c</sup>	243.5 <sup>a</sup>	208.5 <sup>b</sup>	107.0 <sup>c</sup>	212.4 <sup>b</sup>	201.2 <sup>b</sup>	5.10
72	103.4 <sup>c</sup>	271.5 <sup>a</sup>	247.3 <sup>b</sup>	116.7 <sup>c</sup>	245.8 <sup>b</sup>	232.9 <sup>b</sup>	11.8

a,b,c,d. Different letter superscripts in the same row indicate significant difference ( $p \leq 0.05$ ).

CBC = Water buffalo cellulolytic bacteria consortium ( $1.29 \times 10^9$  cells  $\text{mL}^{-1}$ ); TRB = Total ruminal bacteria ( $9.73 \times 10^9$  cells  $\text{mL}^{-1}$ ); Coculture = CBC + TRB; SME = Standard mean error.

With cobra grass, methane ( $\text{CH}_4$ ) production was highest ( $p \leq 0.05$ ) when fermented with TRB at 24 h (Table 3). At 48 and 72 h, the coculture and TRB did not differ ( $p > 0.05$ ) although both were higher ( $p \leq 0.05$ ) than CBC. In contrast,  $\text{CH}_4$  production in the corn stover was highest ( $p \leq 0.05$ ) with TRB at all the evaluated times (Table 3). Overall, the CBC exhibited the lowest  $\text{CH}_4$  production in both cobra grass and corn stover at all the evaluated times (Table 3).

TABLE 3

**Table 3.** Methane ( $\text{CH}_4$ ) volume ( $\text{mL g}^{-1} \text{DM}$ ) accumulated during *in vitro* fermentation of cobra grass and corn stover inoculated with a cellulolytic bacteria consortium from water buffalo, total ruminal bacteria from a cow and a coculture of these communities.

Incubation Time (h)	Cobra Grass			Corn Stover			SME
	CBC	Coculture	TRB	CBC	Coculture	TRB	
24	10.2 <sup>f</sup>	22.5 <sup>b</sup>	25.2 <sup>a</sup>	12.7 <sup>e</sup>	19.0 <sup>c</sup>	16.4 <sup>d</sup>	1.10
48	15.3 <sup>c</sup>	44.4 <sup>a</sup>	42.2 <sup>a</sup>	17.9 <sup>c</sup>	41.7 <sup>a</sup>	37.0 <sup>b</sup>	2.57
72	15.8 <sup>c</sup>	54.1 <sup>a</sup>	53.7 <sup>a</sup>	18.4 <sup>c</sup>	49.6 <sup>a</sup>	42.2 <sup>b</sup>	3.45

a,b,c,d. Different letter superscripts in the same row indicate significant difference ( $p \leq 0.05$ ). CBC = Water buffalo cellulolytic bacteria consortium ( $1.29 \times 10^9$  cells  $\text{mL}^{-1}$ ); TRB = Total ruminal bacteria ( $9.73 \times 10^9$  cells  $\text{mL}^{-1}$ ); Coculture = CBC + TRB; SME = Standard mean error.

No differences ( $p > 0.05$ ) were observed in DMD and NDFD between the coculture and TRB in either substrate; however, overall DMD and NDFD values were higher in the cobra grass than in the corn stover.

The lowest ( $p \leq 0.05$ ) DMD and NDFD values in both substrates were produced by CBC (Table 4). Bacteria count in the cobra grass did not differ between the treatments ( $p > 0.05$ ). In the corn stover, TRB had a higher ( $p \leq 0.05$ ) count than the coculture but did not differ from CBC ( $p > 0.05$ ; Table 4). Average pH in all treatments and substrates was 6.89 and did not differ ( $p > 0.05$ ) between them. Ammoniacal nitrogen ( $\text{NH}_3\text{-N}$ ) concentration did not differ between the coculture and TRB in the corn stover ( $p > 0.05$ ), but was higher in the coculture with cobra grass ( $p \leq 0.05$ ; Table 4).

TABLE 4

**Table 4.** Dry matter and neutral detergent fiber degradation, bacteria count, pH and ammoniacal nitrogen during *in vitro* fermentation of cobra grass and corn stover inoculated with a cellulolytic bacteria consortium from water buffalo, total ruminal bacteria from a cow and a coculture of these communities.

Variable	Cobra Grass			Corn Stover			SME
	CBC	Coculture	TRB	CBC	Coculture	TRB	
DMD	29.65 <sup>c</sup>	70.92 <sup>a</sup>	70.42 <sup>a</sup>	27.86 <sup>c</sup>	59.20 <sup>b</sup>	60.84 <sup>b</sup>	3.70
NDFD	10.67 <sup>c</sup>	67.05 <sup>a</sup>	66.33 <sup>a</sup>	3.94 <sup>d</sup>	50.31 <sup>b</sup>	51.32 <sup>b</sup>	5.75
Bacteria	1.28 <sup>abc</sup>	1.36 <sup>abc</sup>	1.68 <sup>a</sup>	1.19 <sup>bc</sup>	1.08 <sup>c</sup>	1.56 <sup>ab</sup>	0.06
pH	6.93	6.91	6.88	6.95	6.87	6.84	0.05
$\text{NH}_3\text{-N}$	18.26 <sup>c</sup>	21.91 <sup>a</sup>	16.77 <sup>d</sup>	19.35 <sup>bc</sup>	20.50 <sup>ab</sup>	20.04 <sup>b</sup>	0.36

a,b,c,d: Different letter superscripts in the same row indicate significant difference ( $p \leq 0.05$ ).

CBC = Water buffalo cellulolytic bacteria consortium ( $1.29 \times 10^9$  cells  $\text{mL}^{-1}$ ); TRB = Total ruminal bacteria ( $9.73 \times 10^9$  cells  $\text{mL}^{-1}$ ); Coculture = CBC + TRB; DMD = Dry matter degradation; NDFD = Neutral detergent fiber degradation; Bacteria =  $10^9$  cells  $\text{mL}^{-1}$ ;  $\text{NH}_3\text{-N}$  =  $\text{mg dL}^{-1}$   $\text{NH}_3\text{-N}$ ; SME = Standard mean error.

## DISCUSSION

The coculture treatment simulated addition of a cellulolytic bacterial consortium (CBC) to the bacterial community of the cattle rumen, allowing analysis of any changes in cellulosic substrate degradation in response to the addition. The differences in gas production (Table 2) and DMD (Table 4) observed at the tested times can be attributed to the nutrients available in the substrates, efficiency of substrate use by the microorganisms, and microorganism source and density (18). Gas production during the first 24 h responds to the capacity of TRB, CBC or the coculture to ferment substrate cell content. After 24 h, gas production by the coculture in cobra grass was due to the capacity of the cellulolytic bacteria to degrade cellulose, and, unlike with TRB, was related to gas production. Beginning at 48 h, gas production in the corn stover did not differ ( $p > 0.05$ ) between the coculture and TRB, which can be attributed to the type of nutrients in this substrate (Table 1). These differences in gas production between the coculture and TRB in the two tested substrates are due to the corn stover having 8% more NDF and 10% more hemicellulose than the cobra grass (Table 1). The corn stover's higher NDF and hemicellulose contents are related to vegetal cell aging and changes in cell wall composition in which cellulose mixes with hemicellulose and lignin (19). Lower gas production than that observed here for the coculture at 48 h has been reported for *Brachiaria decumbens* inoculated with buffalo ruminal fluid (152 mL gas  $\text{g}^{-1}$  DM) or bovine ruminal fluid (167 mL gas  $\text{g}^{-1}$  DM) and fermented for 48 h (20).

Differences observed in  $\text{CH}_4$  production (Table 3) were due to substrate carbohydrate content (Table 1) (19,23), and inoculate type (18). For instance,  $\text{CH}_4$  production was 33.1 mL  $\text{CH}_4$   $\text{g}^{-1}$  DM at 120 h

incubation in a study of water buffalo ruminal fluid as an inoculate in wheat hay (21). This is lower than production observed here at 72 h for the coculture and TRB treatments. The difference between the two studies is due to wheat hay containing only 42% total digestible nutrients while corn stover contains 59% (22). The coculture produced higher CH<sub>4</sub> production than TRB in the corn stover, probably due to the volatile fatty acids production pattern (19). Cellulolytic bacteria produce acetate and hydrogen as cellulose fermentation products (11,23), and hydrogen is the substrate used by methanogenic Archaea to synthesize CH<sub>4</sub> (24).

The DMD percentages observed here differed from some previous studies. Those for cobra grass were higher than *in vitro* DMD values reported for sheep ruminal bacteria on two substrates: 55.9% for *Nigella sativa* and 54.0% for *Rosmarinus officinalis* (25). However, they were lower than values reported for CBC and similar to those for cocultures (23). In a study using rice hay, NDFD values were 78.4% for buffalo TRB and 71.6% for cattle TRB, with no differences between inocula. This is similar to the lack of difference observed here between TRB and the coculture in both the cobra grass and corn stover. The degradation percentages produced by CBC in the evaluated substrates can be attributed to its need to interact with microorganisms via nutritional interdependence and/or cross-feeding (26), as well as catabolic repression from the presence of glucose and other compounds in the medium, which inhibit enzymatic synthesis (27).

Due to the type of cellulolytic activity in the rumen, fermentation of the structural carbohydrates in cobra grass and corn stover does not change pH (28). The present results support this conclusion (Table 4). Ruminal bacteria, and particularly cellulolytic bacteria, require pH levels near neutral to function correctly (23), because their enzymatic activity is inhibited at levels below 6.0 (29).

The bacteria populations documented in the present results (Table 4) are higher than the 108 bacteria mL<sup>-1</sup> reported for a lyophilized cellulolytic consortium without preservatives (11), and a buffalo and cattle inoculum used to ferment fibrous compounds (2). Variations in bacteria populations are caused by the intraruminal microorganism dynamic, and characterized based on fermented nutrient type (8), and pH and NH<sub>3</sub>-N concentration (30). Medium NH<sub>3</sub>-N concentration influences substrate CP content and degradability (2). However, NH<sub>3</sub>-N concentration in the interactions between treatments and substrates did not differ ( $p > 0.05$ ) between substrates (Table 4) even though CP content in the cobra grass and corn stover differed by four percent (Table 1). The NH<sub>3</sub>-N concentrations and CP contents were higher than reported for ruminal fluid from water buffaloes fed rice hay: 5.5 mg dL<sup>-1</sup> NH<sub>3</sub>-N and 92 g Kg<sup>-1</sup> d<sup>-1</sup> CP (2). Concentrations of NH<sub>3</sub>-N are believed to be directly related to microbial populations since variations in ruminal bacteria depend on NH<sub>3</sub>-N content (29). However, this relationship was not observed in the present data since the higher bacteria counts did not coincide with higher NH<sub>3</sub>-N concentrations (Table 4). Differences in NH<sub>3</sub>-N concentration and bacteria count influence each treatment's capacity to degrade substrates and the efficiency of microbial nitrogen synthesis (2).

The gas production, and dry matter and neutral detergent fiber degradation values for the studied water buffalo cellulolytic bacteria consortia suggest that, when cocultured with bovine ruminal bacteria, they may be an alternative for improving structural carbohydrate fermentation in cobra grass. Coculture did not improve fermentation in the corn stover due to differences between the substrates used to produce the isolated cellulolytic consortium and stover proximate chemical composition. The cellulolytic bacteria consortia studied here require further *in situ* and *in vivo* evaluations to determine their potential use in producing a cellulolytic probiotic.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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